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Systematics and Evolution of Lower Central American Cichlids Inferred from Analysis of Cytochrome b Gene Sequences

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Central American cichlids allied to the genus "*Cichlasoma*" are thought to be of South American ancestry. The group has apparently undergone extensive morphological, ecological, and behavioral differentiation in Central America following colonization. Uncertainties regarding the systematics of the group and the timing of colonization complicate interpretation of the biological history of cichlids in Central America. We determined complete cytochrome b gene sequences for 54 individual cichlids representing 21 species to test hypotheses regarding the time of origin and pattern of diversification of lower Central American cichlids. The data also bear on issues relating to the systematics and taxonomy of heroine cichlids. Our results suggest that cichlids have been in Central America since the middle to late Miocene. Moreover, the data provide evidence of a rapid radiation early in the history of the group. Similar ecomorphological types have evolved multiple times. Inferences of convergent morphological evolution may, in part, explain a lack of concordance between the mitochondrial gene tree and previous inferences of phylogenetic relationships based on observable characteristics. Phylogenetic inferences based on the molecular data provide support for the recognition of "sections" [erected by Regan (1905) and others] as distinct genera and suggest that thorough revision of Central American cichlids is necessary before the extent of biological diversity within the group can be fully appreciated.

Key Words: **cytochrome b; Central America; *Cichlasoma;* systematics; adaptive radiation**

INTRODUCTION

Central American cichlids allied to the genus *Cichlasoma* exhibit great morphological, ecological, and behavioral differentiation. There are over 75 described species of *Cichlasoma* (Regan, 1905; Miller, 1966; Bussing, 1985). The diversity of Central American cichlids is more remarkable when you consider that most other genera of freshwater fishes derived from South America are represented in Central America by a single species or a handful of species. Although the species diversity of Central American cichlids does not match that present in Africa (Meyer, 1993), the ecomorphological diversity observed among sympatric *Cichlasoma* species approaches that observed among more phylogenetically divergent cichlids (Winemiller *et al.*, 1995), suggesting that Central American cichlids underwent a recent explosive radiation similar to that inferred for cichlids of the East African Great Lakes (Meyer *et al.*, 1990; Meyer, 1993; Sturmbauer and Meyer, 1993; Moran *et al.*, 1994). Based on a phylogenetic hypothesis, Winemiller *et al.* (1995) concluded that Central American fluvial cichlids have undergone a more rapid rate of niche diversification than fluvial cichlid faunas from other parts of the world (South America and Africa).

Although it is likely that Central American cichlids diversified relatively recently, there are several possible alternative hypotheses for the high species diversity of *Cichlasoma* in Central America. Bussing (1985) hypothesized that the large number of species and extensive range of *Cichlasoma* in Central and Middle America could be explained by an ancient colonization of Central America from South America, perhaps as early as the late Cretaceous or Paleocene. Moreover, Bussing (1985) suggested that the early pool of colonists from South America may have included multiple lineages of cichlids. Myers (1966) hypothesized that *Cichlasoma* colonized Central America relatively recently and suggested an early Miocene date of arrival from South America based on the occurrence of a Miocene fossil of *Cichlasoma* from the Antillean island of Hispaniola. Myers (1966) further suggested that

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cichlids diversified rapidly to fill the "ostariophysan vacuum" that existed in Central America prior to colonization of freshwater fishes from South America. Finally, the genus may have colonized Central America following the rise of the Isthmus of Panama in the Pliocene. This could have been accomplished by multiple distinct lineages that were present in northwestern South America during this time or the extant species may be derived from a single colonist followed by an explosive radiation. The extensive distribution of the genus (the northern limit is Texas) can be explained by their ability to tolerate changes in salinity, suggesting that many species (or their ancestral stocks) may have migrated along coastlines.

Tests of hypotheses regarding the timing and the pattern of diversification require robust inference of evolutionary relationships among representative lineages. The sheer numbers of *Cichlasoma* species, coupled to the considerable uncertainty regarding their systematic status (Kullander, 1983, 1996, and pers. comm.), suggest that it would be premature to attempt exhaustive tests of alternative phylogenetic hypotheses for *Cichlasoma*. Instead, we use molecular information about evolutionary relatedness of species to test alternative hypotheses about the timing of colonization of Central America from South America and to test the hypothesis that *Cichlasoma* underwent an explosive radiation following colonization of an ostariophysan vacuum.

Background Systematics

Recent research has significantly altered our view of the genus *Cichlasoma*. Kullander (1983) recognized only 12 species in the genus *Cichlasoma*, all of which are distributed on the South American continent. Furthermore, Kullander's *Cichlasoma* are cichlasomines [large-scaled cichlasomines or Group B cichlasomines (Stiassny, 1991, p. 31)], whereas all Central American *Cichlasoma* are heroines [small-scaled cichlasomines or Group A cichlasomines (Stiassny, 1991, p. 31)] (Kullander, 1996). Thus, the nomenclature of the Central American heroines is in disarray. Kullander (1983, p. 270) recommended ". . . the continued recognition of Central American groups as sections [following Regan (1905)], especially as some, like *Theraps* and *Amphilophus*, are not well defined." Nonetheless, the working hypothesis of most cichlid systematists elevates Regan's sections to genera. Thus for the remainder of this paper we will treat Regan's 1905 sections, including modifications by others (Bussing and Martin, 1975; Kullander, 1983, 1986, 1996; Allgayer, 1988; R. Miller, pers. comm.), as genera.

Following Kullander's (1983) discussion of Central American "*Cichlasoma*" sections and genera, we recognize the following heroine "*Cichlasoma*" genera in Costa Rica: *Amphilophus*, *Archocentrus*, *Hypsophrys* (= *Copora*), *Herotilapia*, *Neetroplus*, *Parachromis*, *Paraneetroplus*, and *Tomocichla*. Representatives of all genera in Costa Rica are included in this study. In addition, we have included two additional heroine genera from Colombia and Ecuador: *Caquetaia* and *Heros*, respectively. *Cichlasoma boliviensis* from Peru is a true *Cichlasoma* and thus the only cichlasomine representative in our study. From here on we will refer to the different cichlid groups by the generic names introduced here and we will reserve "*Cichlasoma*" for reference to the "nomenclature limbo" that continues to plague cichlid systematics. Presently, the systematic relationships of Central American heroine cichlid genera are unresolved, as are the relationships to South American heroine and cichlasomine genera.

In this paper we present a phylogenetic analysis of Costa Rican cichlids. We have specifically focused on Costa Rican cichlids because the taxonomy is well known and the geographic ranges of species are well documented. We have included a few species from South America to provide perspective on the relationship between Costa Rican heroine genera; however, the focus of analysis remains on the Costa Rican fish. Our results indicate that the cytochrome b gene is well suited for the investigation of the timing and the pattern of diversification within genera of Central American fishes and that molecular data point to greater diversity than has been appreciated with morphological studies alone. Analysis of the cytochrome b gene sequences is consistent with a hypothesis of invasion of Central America by a single cichlid lineage in the middle Miocene, followed by a rapid radiation of lineages; however, the hypothesized monophyly of Central American cichlids remains tentative. In addition, the data were unable to reject hypotheses of monophyly for some genera, and in other cases, hypotheses of monophyly of recognized genera were refuted. These findings emphasize the necessity of systematic study and taxonomic revision of Central American cichlids.

MATERIALS AND METHODS

Table 1 lists the species included in this study. Fish were collected using a Smith-Root electrofisher, cast nets, or seines, and all Costa Rican taxa were identified in the field by Dr. William Bussing (University of Costa Rica) (Fig. 1). The identifications of most voucher specimens have been confirmed by Dr. Sven Kullander (Swedish Museum of Natural History). Gill arches and small pieces of muscle were preserved at ambient temperature in a saturated salt solution (NaCl) of dimethyl sulfoxide and disodium ethylenediaminetetraacetate (EDTA) (Seutin *et al.*, 1991). The whole fish was labeled with a number corresponding to the tissue sample, preserved in buffered Formalin, and later transferred to 70% ethanol. Samples were collected, exported, and imported under appropriate permits on a series of trips to the following countries: Costa Rica

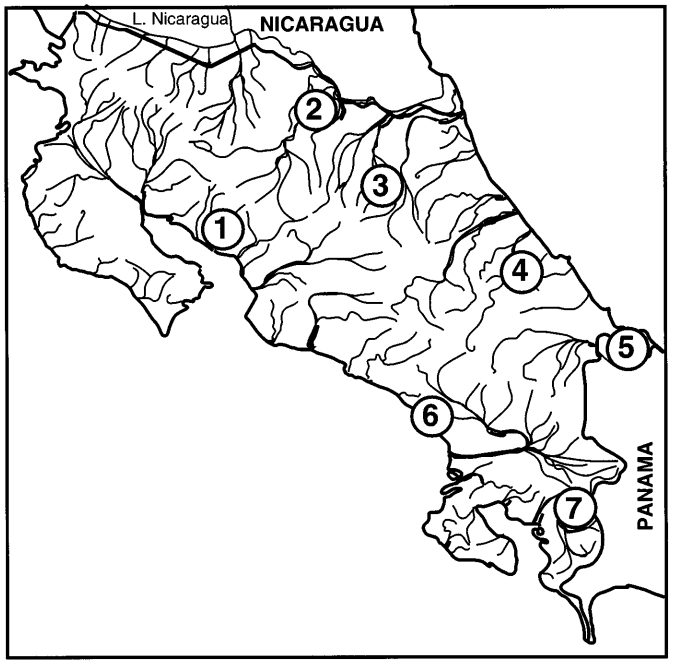

FIG. 1. Map of Costa Rica showing locations of drainages where we collected cichlids used in this study. 1, Barranca; 2, San Juan; 3, Sarapiqui; 4, Matina; 5, Sixaola; 6, Terraba; and 7, Coto.

(January 1994), Colombia (March/April 1994), Ecuador (October 1992), and Peru (July 1993). All voucher specimens except the Ecuador samples have been accessioned into a small museum housed at the Naos Laboratories of the Smithsonian Tropical Research Institute. The vouchered Ecuador specimens are located in Nacional Politechnica of Quito.

We analyzed two to six individuals for 16 of 22 species and included individuals from separate drainages when possible (Table 1). Small pieces of muscle or gill (0.1 to 0.5 g) were used to isolate total genomic DNA using a phenol/chloroform extraction process (Sambrook *et al.*, 1989; Palumbi *et al.*, 1991). The tissues were digested overnight in a proteinase K buffer (20 µg/ml proteinase K, 20 mM EDTA, 10 mM Tris, pH 7.5, and 1% sodium dodecyl sulfate) maintained at 55°C in a circulating water bath. DNA was extracted twice with phenol: chloroform:isoamyl alcohol (25:24:1 by volume), followed by a chloroform:isoamyl alcohol (24:1) extraction. Next, DNA was precipitated overnight (4°C) using 7.5 M ammonium acetate and 100% ethanol. Following precipitation, samples were washed with 70% ethanol, dried, and resuspended in 100 µl TE (10 mM Tris, pH 8.0, 1 mM EDTA). Typically, 1 µl of this solution was used to provide a DNA template for the polymerase chain reaction (PCR).

The entire cytochrome b gene was PCR amplified using primers in the flanking glutamine (GluDG.L TGACT TGAAR AACCA YCGTT G; Palumbi *et al.*, 1991) and threonine (Cb6b.H GGAAT TCACC TCTCC GGTTT ACAAG AC) tRNA genes. Amplifications were carried out in a Perkin–Elmer DNA Thermalcycler 480. Double-stranded DNA was synthesized in 50-µl reactions [1 µl DNA (~15–20 ng), 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.01% NP-40, 2 mM each dATP, dCTP, dGTP, and dTTP (Sigma), 10 mM GluDG.L, 10 mM Cb6b.H, and 0.25 µl of Amplitaq polymerase (Perkin–Elmer)]. DNA was amplified using a step-cycle profile: denaturation at 94°C for 40 s, primer annealing at 50°C for 40 s and primer extension at 72°C for 1 min, 30 s, repeated for 30 cycles.

The 1.3-kb PCR products were electrophoretically separated from unincorporated primers and dNTPs by electrophoresis in 1.5% low-melting-point agarose gels run in Tris-acetate buffer (pH 7.8) containing ethidium bromide (1 µg/ml). The single amplification product was cut from the gel and extracted using the Gene Clean II kit from Bio 101, Inc. The purified mtDNA was resuspended in 25 µl of ddH_2O, of which 7 µl was used as a template in a cycle sequencing reaction using the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.) and primers GluDG.L, Cb6b.H, and Cb2r1 (TATGT CCTTC CATGA GGRCA AAT). We used a Gene Amp PCR System 9600 (Perkin–Elmer) and the following cycling conditions: 96°C preheat, then 25 cycles of 96°C for 15 s, 50°C for 1 s, and 60°C for 4 min. The cycle sequencing product was purified over Centrisep columns filled with 780 µl G-50 Sephadex. Samples were dried and resuspended in 3.5 µl of a 5:1 deionized formamide:blue dextran/EDTA (pH 8.0) solution, denatured at 90°C for 2 min, and loaded into 6% acrylamide gels. Gels were run on an Applied Biosystems 373A DNA Sequencer for 12 h, with 28 W constant power, and we typically collected 375 to 450 nucleotides per reaction.

Following gel electrophoresis, samples were tracked through the middle of each lane to optimize the read. After tracking, gels were analyzed using 373 DNA Sequencing Analysis software (2.01s, Applied Biosystems, 1992) to generate a chromatogram file. Chromatograms were imported into SeqEd (1.0.3s; Applied Biosystems, 1992) for base verification. The base sequence was checked for miscalls due to bad base spacing, overfluorescence of particular dye nucleotides, or the masking of a C nucleotide by a G nucleotide. Following the cleaning of each primer sequence for an individual, these sequences were then pieced together and exported into a MacVector 4.5.3 file.

All phylogenetic analyses were conducted using PAUP* test Version 4.0d55, written by David L. Swofford, except where noted. Nucleotide composition was examined for variable sites, and the χ^2 test for homogeneity of base frequencies was done for all positions and for only third codon positions. Only single representatives of divergent genera were included in homogeneity tests to limit the correlation due to shared ancestry. Compositional heterogeneity was also examined by

estimating the compositional distance between pairs of taxa using the equation

$$D = \Sigma_i(p_i - q_i)^2/\Sigma[2P_i(1 - p_i)/n],$$

where the frequencies of the four bases (i = G, A, T, C) in two sequences being compared are p_i and q_i, respectively; P_i is the average frequency of the ith base across all individuals being compared; and n is the number of nucleotides in the comparison. Pairwise compositional distances were subjected to least-squares cluster analysis to identify groups (Martin, 1995).

MacClade (Maddison and Maddison, 1992) was used to determine the number of character state changes per site for minimum-length topologies. Distributions of character state changes per site were compared to expected Poisson and negative exponential distributions using standard contingency tests. In some cases, the number of sites in the zero-hits-per-site class was modified to increase the match between observed and expected distributions. In all cases, contingency tests were performed for variable sites (invariant sites were eliminated). For all pairwise comparisons, the numbers of transitions and transversions and the number of substitutions per site were determined. Rate heterogeneity across sites was estimated using the methods of Yang (1994) and Sullivan *et al.* (1995) implemented with PAUP.

Minimum-length trees were determined using weighted parsimony in which transversions were weighted more than transitions and the weightings varied across codon positions to reflect differences in rates across sites. Various weighting schemes were employed, including equal weights across sites and for different substitution types, but results from only two substitution models are reported: a model using maximum transition to transversion ratios at each codon position (22:7:10), where the numbers indicate the weights applied to transversions relative to transitions and the three numbers indicate weightings applied to first, second, and third codon positions, respectively; and a model in which transitions were eliminated from third codon positions and first and second codon positions were weighted according to maximum transition to transversion ratios. In all cases, the heuristic search option with TBR branch swapping and random addition of taxa (20 replicates) was used as the tree-finding algorithm. Bootstrap analysis employed 200 pseudoreplicates. *A priori* hypotheses of monophyly were evaluated using Wilcoxon sign-rank tests (Templeton, 1983).

Genetic distances were corrected for multiple substitutions using a variety of algorithms: (1) Kimura's two-parameter method for all substitutions (Kimura, 1980), (2) the Jukes–Cantor correction on only transversion substitutions (data coded as purines and pyrimidines), (3) a maximum-likelihood HKY substitution model (Hasegawa *et al.*, 1985) assuming a transition to transversion ratio of 10 and a gamma shape parameter of 0.3, and (4) log determinants. Topologies were generated from matrices of corrected genetic distances using the neighbor-joining algorithm (Saitou and Nei, 1987). Hasegawa–Kishino log-likelihood tests assuming a HKY + gamma substitution model (described previously) were performed on the four genetic distance trees to evaluate whether alternative topologies provided significantly better explanations of character covariation across taxa.

RESULTS AND DISCUSSION

The Gene and DNA Sequence Variation

The complete mtDNA cytochrome b gene sequence (1137 bp corresponding to 379 encoded amino acids) was determined for 54 cichlid fishes, representing 12 genera and 21 species (Table 1). All sequences begin with an ATG start codon and end with TAA or TAG. Across all taxa there are 446 variable sites.

Nucleotide composition is typical of a mitochondrial gene (data not shown; see also Roe *et al.*, 1997). Differences in composition across species are most evident at the third codon position. Base frequencies are homogeneous across all variable sites ($\chi^2 = 33.5$, $df = 27$, $P = 0.18$) but not at variable third codon positions ($\chi^2 = 42.7$, $df = 27$, $P = 0.03$) (Table 2A). This is significant because 285 of the 363 variable sites across the 10 genera examined are third codon positions. We further examined the base frequency heterogeneity at third codon positions by estimating compositional distance for all pairwise comparisons among distinct in-group genera (Table 2B). Cluster analysis indicates that there are two distinct groups and suggests that at third codon positions there is some indication of heterogeneity in substitution rates among the four nucleotides across taxa. The difference between groups reflects alternative preference for either C or T nucleotides (Table 2A), indicating that this problem can be alleviated by eliminating or downweighting transitions at third codon positions.

Estimates of the gamma shape parameter for rate heterogeneity across sites were 0.25 and 0.34 (using the methods of Yang and Sullivan *et al.* (1995), respectively), indicating a relatively high level of among-site substitution rate heterogeneity. Patterns of rate heterogeneity across first and second codon positions were similar (Fig. 2). The distribution of substitutions per site at third codon positions most closely approximated a Poisson distribution (Fig. 2B), even though the distribution of transversions per third codon position is best described by a negative exponential function (Fig. 2A). These results suggest that selection impinges on all nucleotide substitutions at first and second codon positions. At third codon positions the pattern of substitution is heterogeneous: transversions appear to be sub-

ject to selection, whereas transitions conform closely to predictions for "neutral" sites. These patterns of constraint match predictions of codon degeneracy and resemble patterns described for other proteins and for the cytochrome b gene in other taxa (Irwin *et al.*, 1991; Martin, 1995), and they suggest that trees derived from

TABLE 1

List of Species Included in this Study, with Drainage Identity and Accession Numbers for Specimens and Tissues

Genus	Species	Drainage	Accession No.
Amphilophus	*alfari*	Barranca, Costa Rica	STRI-2109[a]
		Sixaola, Costa Rica	STRI-0205[a]
		Matina, Costa Rica	STRI-1279[a]
		San Juan, Costa Rica	STRI-1241[a]
Amphilophus	*diquis*	Terraba, Costa Rica	STRI-2066[a]
			STRI-2067
Amphilophus	*longimanus*	San Juan, Costa Rica	STRI-2133[a]
Amphilophus	*rostratum*	San Juan, Costa Rica	STRI-1235[a]
Amphilophus	*rhytsma*	Sixaola, Costa Rica	STRI-0213[a]
			STRI-0214
Archocentrus	*centrarchus*	San Juan, Costa Rica	STRI-2137[a]
			STRI-2138
Archocentrus	*nigrofasciatum*	Sixaola, Costa Rica	STRI-0209[a]
			STRI-0210
		Barranca, Costa Rica	STRI-2106[a]
			STRI-2107
		San Juan, Costa Rica	STRI-2144[a]
			STRI-2145
Archocentrus	*sajica*	Terraba, Costa Rica	STRI-2069[a]
			STRI-2070
Archocentrus	*septemfasciatum*	Sixaola, Costa Rica	STRI-0207[a]
			STRI-0208
Archocentrus	*myrnae*	San Juan, Costa Rica	STRI-1236[a]
			STRI-1237
Caquetaia	*kraussii*	Atrato, Colombia	STRI-1523[a]
Caquetaia	*umbiferum*	Atrato, Colombia	STRI-1527[a]
Herotilapia	*multispinosa*	Barranca, Costa Rica	STRI-1204[a]
		San Juan, Costa Rica	STRI-2148
Nandopsis	*atromaculatum*	Baudo, Colombia	STRI-1404[a]
			STRI-1405
Parachromis	*dovii*	San Juan, Costa Rica	STRI-2125[a]
Parachromis	*loisellei*	San Juan, Costa Rica	STRI-2124[a]
			STRI-2126
Neetroplus	*nematoplus*	San Juan, Costa Rica	STRI-2142[a]
Hypsophrys	*nicaraguensis*	San Juan, Costa Rica	STRI-2146[a]
			STRI-2147
		Matina, Costa Rica	STRI-1278[a]
Paraneetroplus	*sieboldii*	Coto, Costa Rica	STRI-1161[a]
			STRI-1162
		Barranca, Costa Rica	STRI-2104[a]
			STRI-2105
Tomocichla	*tuba*	San Juan, Costa Rica	STRI-2131[a]
			STRI-2132
Heros	*appendiculatus*	Cuyabeno, Ecuador	ClseEC1[a]
			ClseEC2
Cichlasoma	*boliviensis*	Manu, Peru	STRI-369[a]
			STRI-370

[a] Individuals included in the analyses. For most species and geographic locations, two individuals were surveyed to confirm sequence and species identity.

TABLE 2

A Nucleotide Composition at Variable Third Codon Positions for Representatives of 10 Genera

	A	C	G	T
Parachromis	0.2455	0.5017	0.0107	0.2420
Hysophyrs	0.2132	0.4685	0.0420	0.2760
Archocentrus	0.2070	0.4491	0.0350	0.3087
Paraneetroplus	0.2299	0.4704	0.0418	0.2578
Amphilophus	0.2132	0.4825	0.0209	0.2832
Heros	0.2369	0.4216	0.0314	0.3101
Caquetaia	0.1866	0.5528	0.0528	0.2077
Nandopsis	0.2202	0.5454	0.0349	0.1993
Tomocichla	0.2106	0.5296	0.0418	0.2125
Herotilapia	0.2049	0.5406	0.0424	0.2120
Average	0.2168	0.4962	0.0354	0.2509

B Matix of Pairwise Compositional Distances at Variable Third Codon Positions for Representatives of Distinct In-group Genera

		1	2	3	4	5	6	7	8	9
1	*Parachromis*									
2	*Hysophyrs*	0.95								
3	*Archocentrus*	**2.08**	0.33							
4	*Paraneetroplus*	0.54	0.13	0.78						
5	*Amphilophus*	0.72	0.15	0.43	0.33					
6	*Heros*	**2.58**	0.88	0.36	**1.15**	**1.13**				
7	*Cacquetaia*	**2.02**	**2.76**	**4.73**	**2.47**	**2.79**	**6.93**			
8	*Nandopsis*	**1.11**	**2.60**	**4.67**	**2.01**	**2.54**	**6.24**	0.35		
9	*Tomocichla*	0.85	**1.70**	**3.44**	**1.30**	**1.71**	**4.92**	0.28	0.12	
10	*Herotilapia*	**1.13**	**2.05**	**3.88**	**1.67**	**2.02**	**5.62**	0.13	0.10	0.03

Note. Values in excess of 1 indicate significant compositional divergence (Gillespie, 1986) and are shown in boldface.

substitution models that approximate the heterogeneity of substitution rate across sites will yield the best phylogenetic inferences (Hillis *et al.*, 1994).

Patterns of nucleotide substitution in cichlid fishes are very similar to patterns observed for other fishes (Martin, 1995) and vertebrates (Irwin *et al.*, 1991). Rates of transition substitutions are at least an order of magnitude greater than transversion substitution rates (Fig. 3). Figure 3A suggests that multiple substitutions at a site have not fully obliterated the record of transitions over the range of divergences surveyed (the slope of the second order regression equation is positive for all values of x). Nevertheless, transition to transversion ratios decline precipitously and approach the asymptote of approximately 2 for comparisons among the most divergent taxa (Fig. 3B). Thus, transitions do not appear to provide much phylogenetic information regarding relationships between lineages whose divergence is more than approximately 0.1 substitutions/site.

Phylogenetic Hypotheses

Weighted parsimony analysis, using the 22:7:10 scheme, yielded a single minimum-length tree with few

well-supported clades (Fig. 4). There is good support for: (1) a diverse Costa Rican clade (*Archocentrus, Parachromis, Paraneetroplus, Neetroplus and Hypsophyrs*), (2) a sister taxa relationship between *Neetroplus and Hypsophyrs*, (3) a clade containing three species of *Archocentrus* (*A. centrarchus, A. septemfasciatum*, and *A. nigrofasciatum*), (4) a close relationship between *A. septemfasciatum* and *A. nigrofasciatum*, and (5) the monophyly of *Amphilophus* and of relationships among species of *Amphilophus*. This tree is significantly different from the four minimum-length trees generated with the same substitution model at first and second codon positions but with transitions at third codon positions omitted (trees not shown) when Wilcoxon sign-rank tests are performed using the former weighting scheme (Table 3). By contrast, trees generated using the alternative weightings are not significantly different when evaluated using the latter weighting scheme (Table 3). There are two noteworthy differences between the trees generated using alternative weightings. First, in trees generated without third position transitions, *A. centrarchus* moves to the base of the clade consisting of *Archocentrus, Paraneetroplus*, and others. Second, removal of third position transitions eliminates phylogenetic information about relationships within *Amphilophus*. We interpret the bootstrap scores as providing the best estimate of phylogenetic signal in the data, and we entertain each of the minimum-length topologies (Fig. 4 and the trees generated without third position transitions) as alternative hypotheses of phylogenetic relationships.

Neighbor-joining topologies were determined from matrices of genetic distances corrected for multiple hits using different methods (for an example, see Fig. 5A).

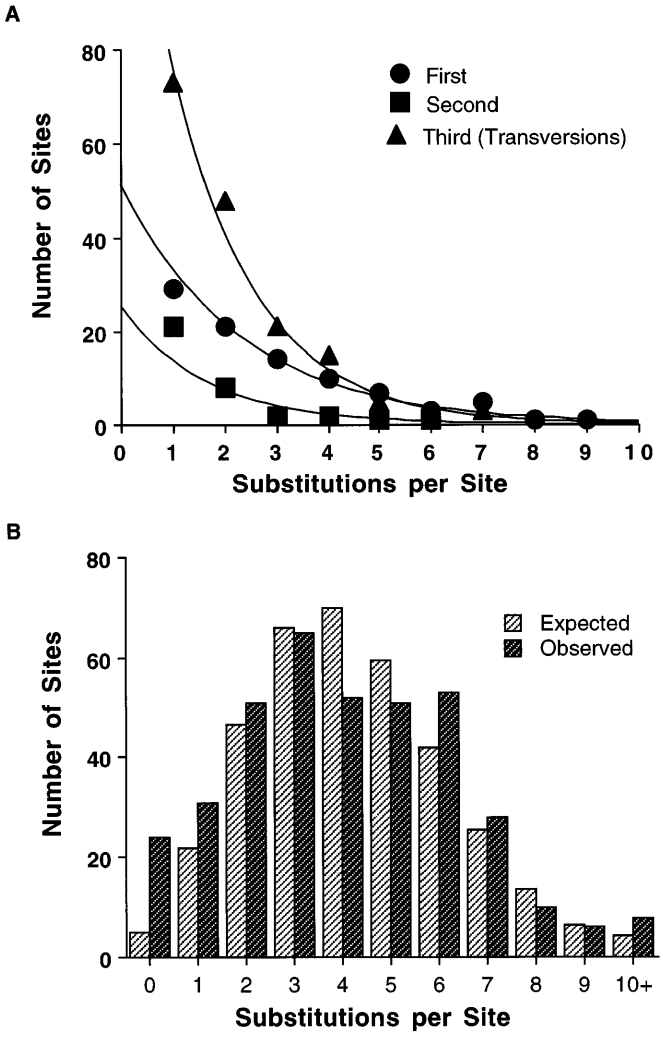

FIG. 2. (A) Negative exponential distributions of variation for all substitution types at first and second codon positions and for transversions at third codon positions. (B) Histogram of the number of substitutions per site at third codon positions. Expected values were determined by iterative elimination of the zero substitution sites until a minimum χ^2 was obtained (see Martin, 1995). For sites with one or more substitutions, $\chi^2 = 8.0$, $P = 0.53$. Inclusion of the zero-hits sites inflates χ^2 to 19.71, $P = 0.032$, indicating significant differences in the distributions.

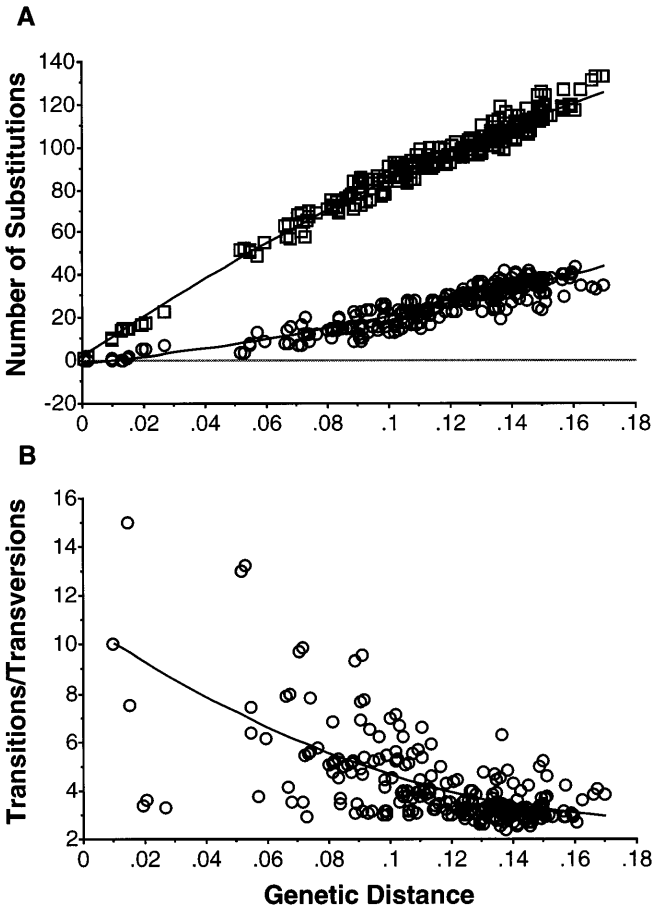

FIG. 3. (A) Plot of the number of transitions (open squares) and transversions (open circles) against the corrected genetic distance (substitutions per site) for the complete cytochrome b gene. (B) Ratio of the number of transitions to the number of transversions plotted against corrected genetic distance.

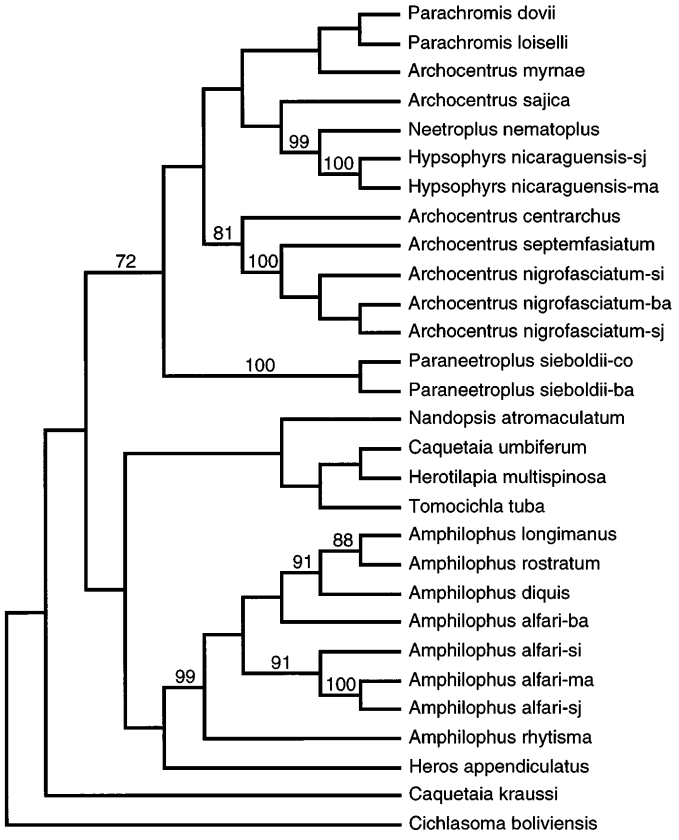

FIG. 4. Minimum-length topology generated using 22:7:10 weighted parsimony. Bootstrap values are provided for clades found in more than 50% of the bootstrapped trees.

The monophyly of *Amphilophus*, the close relationship between *A. septemfasciatum* and *A. nigrofasciatum*, and the sister taxa association of *Hypsophrys* and *Neetroplus* are all supported by relatively long branches. In addition, all four trees contain a group consisting of *Paraneetroplus*, *Archocentrus*, *Parachromis*, *Hypsophyrs*, and *Neetroplus* (Fig. 5B). This large clade is also evident in the maximum-parsimony bootstrap tree (Fig. 4). Otherwise, the relationships among most genera are unresolved, and this presumably reflects an abundance of short internodes, a feature common to all distance-based topologies. Short internodes lend little support to any groupings (other than the well-supported clades mentioned) that may be common among the neighbor-joining and minimum-length topologies and also suggest that differences in topology among trees (see Fig. 5B) determined under different sets of assumptions are not significant (Table 4).

TABLE 3

Wilcoxon Sign-Rank Tests of Alternative Hypotheses

Substitution model	Tree	Length	*Z*	*P*[a]
22:7:10	22:7:10	3843	Best	
	22:7:Tvs only	3971	3.04	0.0023
		3870	2.99	0.0028
		3978	3.27	0.0011
		3879	3.31	0.0009
22:7:Tvs only	22:7:10	742	0.40	0.6884
	22:7:Tvs only	719	Best	

[a] Approximate probability of getting a more extreme test statistic under the null hypothesis of no difference between the two trees (two-tailed test).

Overall, the analysis indicates that Costa Rican heroine cichlids are well differentiated, but are unfortunately refractory to phylogenetic inference using cytochrome b gene sequences. Lack of resolution of relationships is probably symptomatic of a radiation of lineages in a period of time too brief to allow for the accumulation of synapomorphies. Alternatively, lack of resolution may be symptomatic of multiple hits, suggesting that cytochrome b may not be ideally suited for inferring the evolutionary history of Central American cichlids (Meyer, 1994). While this is certainly true for the transition substitutions at third codon positions, there is not a strong signal of saturation for transversion substitutions. Moreover, all of the distance methods yielded quantitatively similar results, suggesting that methods designed to alleviate problems associated with multiple hits did not improve hierarchical signal in the data. Finally, compositional heterogeneity at third codon positions may also have conspired to reduce hierarchical signal in the data.

Pattern and Timing of Diversification

Distribution of pairwise Kimura-corrected genetic distances for 24 distinct Costa Rican taxa surveyed is unimodel, with a pronounced left skew (Fig. 6A). When the taxa are pruned so that only representatives of distinct genera are included, the distribution of pairwise distances is unimodal and highly leptokurtotic, suggestive of a diversification of lineages in a brief period of time relative to the rate of nucleotide substitution (Fig. 6B). This is supported by the abundance of short internode lengths evident in the neighbor-joining trees (see Figs. 5A–5D), regardless of the method used for correcting for multiple hits. Thus, the hypothesis that most genera originated at the same point in time is not refuted by the data.

When did Costa Rican cichlid genera originate? If we use the rate of cytochrome b evolution estimated for marine fishes (1–1.2% sequence divergence per million years; Bermingham *et al.*, 1997), the maximum time of divergence between the two most distinct Costa Rican cichlids surveyed is ≈15–18 million years ago (mya) (genetic distances were corrected for multiple substitutions using Kimura's method). Identification of a Hispaniola fossil *Nandopsis* from the Miocene (Tee-Van, 1935) accords well with the estimated date of divergence among Central American heroine cichlids. These

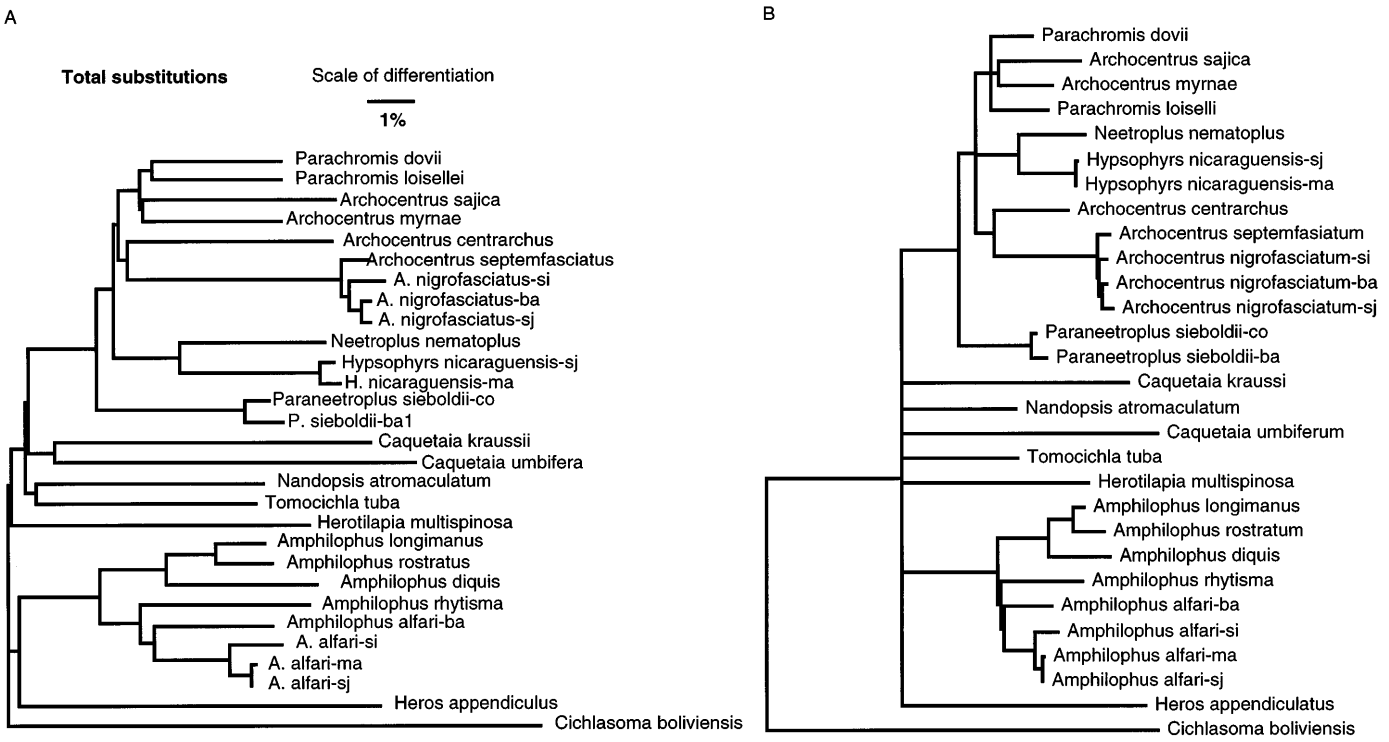

FIG. 5. (A) Topology derived by neighbor-joining cluster analysis of genetic distances corrected for multiple hits using Kimura's two-parameter model. (B) Majority rule consensus tree of four neighbor-joining trees generated using different sets of assumptions and substitution types (see Materials and Methods for details). Branch lengths were estimated using maximum likelihood assuming a HKY substitution model with a transition to transversion parameter of 10 and a gamma shape parameter of 0.3.

dates are at odds with a recently published study of Caribbean and Central American *Rivulus* in which Central American species are hypothesized to have diverged at least 40–46 mya (based on Kimura's corrected genetic distances of cytochrome b and 12S rRNA sequences; Murphy and Collier, 1996). Central American and Caribbean cichlids and *Rivulus* share broadly overlapping distributions, have similar ecological characteristics, and are thought to have originated in South America; thus, if cichlids and *Rivulus* colonized Central America at approximately the same time, the threefold difference in estimated age underscores the uncertainty shrouding the temporal scale of diversification of Central American freshwater fishes. It is likely, however, that the disparity reflects differences in calibration of molecular clocks. Murphy and Collier (1996) used the hypothesized late Cretaceous (70–80 mya) breakup of the proto-Antilles arc and the sequence divergence between Antillean and Central American fish to calibrate the rate of sequence evolution for estimating the divergence time among Central American species. We suspect that the divergence between Central American and Antillean fish occurred much more recently than the late Cretaceous. Moreover, pairwise genetic distances among Central American *Rivulus* are very similar to distances estimated among Costa Rican cichlids, suggesting that cichlids and rivulines may have diversified coincidentally.

TABLE 4

Hasegawa–Kishino Log-Likelihood Test Results for Neighbor-joining Trees

Distance matrix	$-\ln L$	Diff. $-\ln L$	SD	T	P
Maximum likelihood	7756.03	Best			
LogDet	7761.34	5.31	14.5	0.37	0.71
Kimura (all substitutions)	7761.81	5.79	14.2	0.41	0.68
Kimura (only transversions)	7763.45	7.42	15.8	0.47	0.64

Note. HKY model assumed a transition to transversion ratio of 10 and a gamma shape parameter of 0.3.

The great diversity of heroine cichlids in Costa Rica can be explained by the colonization of Central America by multiple genera after the rise of the Isthmus of Panama sometime during the late Pliocene or by the invasion of a single (or a few closely related) lineages sometime before the Pliocene emergence of the Isthmus. The extensive geographic range of heroine cichlids in Central and Middle America relative to other fishes of southern origin argues in favor of an early colonization. Bussing (1985) hypothesized a Paleocene colonization of Central America by South American cichlids, whereas Myers (1966) proposed an early Miocene colo-

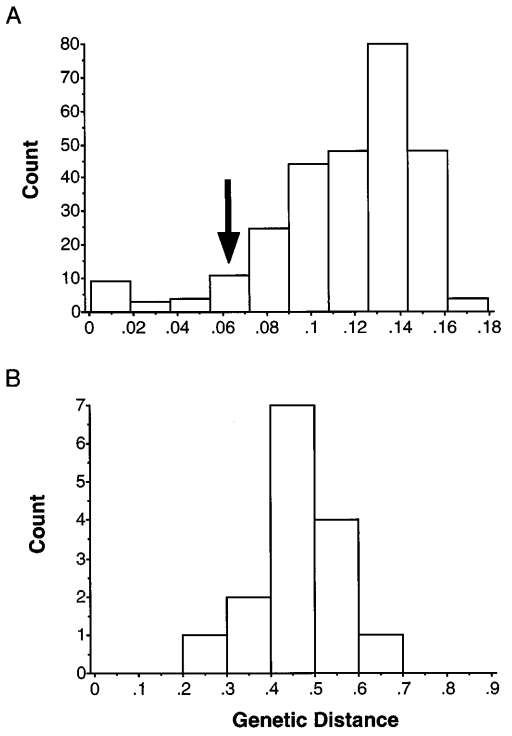

FIG. 6. (A) Distribution of corrected genetic distances at all positions for the 24 distinct Costa Rican lineages surveyed in this study (see Table 1). Arrow marks the average divergence between geminate pairs of species of marine fish thought to have separated 3–4 mya (Bermingham *et al.*, 1997). (B) Distribution of corrected genetic distances at third codon positions for all pairwise comparisons among single representative species of distinct Costa Rican genera. Note the unimodal, leptokurtotic distribution.

nization based, in part, on the presence of the Miocene fossil *Nandopsis* from Hispaniola; however, the ability of many cichlids to tolerate saline conditions suggests that they may be capable of rapid dispersion along coastlines, making it possible that they invaded Central American following the disappearance of the Bolivar seaway in the mid Pliocene. If along-shore dispersal is common, then we would not expect to see significant genetic divergence between populations inhabiting adjacent drainages (i.e., among populations of *Amphilophus alfari*). Thus, we favor the hypothesis of an early (possibly early to middle Miocene) invasion of heroine cichlids into Central America.

Systematics of Central American Heroine Cichlids

The systematics of heroine cichlids is currently under revision (S. Kullander, pers. comm.). Stiassny (1991) recognized large- and small-scaled cichlasomines. Kullander (pers. comm.) suggested restricting cichlasomines to Stiassny's large-scale group and using the term heroines for Stiassny's Group A (small-scaled cichlasomines). The two groups can be distinguished by predorsal scale pattern (Kullander, 1983) and egg-laying behavior (Stiassny, 1991). Synapomorphies of the heroines include aspects of the articulation of the palatine with the vomer, five or more anal-fin spines, and the shape of the anterior teeth (Kullander, 1996). Cytochrome b gene sequences of cichlasomines (represented by *Cichlasoma*) and heroines (including the South America genus *Heros*) are highly divergent, confirming that the morphological difference between these groups is matched by a clear genetic distinction.

Within the heroines, recognized genera are well differentiated (Fig. 5). The relationships among the genera remain elusive, however, with two exceptions: *Hypsophyrs* and *Neetroplus* unambiguously share a common ancestor (Figs. 4 and 5). This close sister-taxa relationship of *Hypsophyrs* and *Neetroplus* contradicts hypotheses of relationships based on morphological characters (Stiassny, 1991; Kullander, pers. comm.). In addition, the data suggest that *Paraneetroplus, Archocentrus, Parachromis, Hyposphyrs,* and *Neetroplus* form a monophyletic group that is distinct from all other genera examined.

Although we were unable to find strong support for intergeneric relationships, the data do permit refutation of previously hypothesized relationships. Based on similarity of form, Bussing (1975) hypothesized that *Tomocichla tuba* and *Paraneetroplus sieboldii* were closely related sister taxa; however, this hypothesis is not supported by the molecular data. *Tomocichla* tends to group with *Nandopsis* (although not consistently). Interestingly, both of these genera are unique in having only four anal spines, whereas most other heroine (small-scaled) cichlids have five or more anal spines (Kullander, pers. comm.). In addition, tooth forms of *Tomocichla* and *Nandopsis* are similar, and both are distinct from the *Paraneetroplus–Hypsophyrs–Neetroplus* clade (Kullander, pers. comm.). Bussing (1985, p. 169) also suspected that *Am. alfari* and *Am. diquis* and *A. sajica* and *A. nigrofasciatum* were sister taxa, respectively, which diverged from a common ancestor as a result of the emergence of the Cordillera in the Pliocene. Although these pairs of taxa may have diverged as a result of the rise of the Cordillera, the level of genetic divergence between the respective pairs suggest that the divergence event happened in the Miocene; moreover, in neither case are these pairs of species sister taxa.

The molecular data indicate that taxonomic revision of some genera may be necessary. First, *Archocentrus* is paraphyletic in the inferred topologies of relationships. If *Archocentrus* is constrained to be monophyletic, the resultant distribution of changes per site is not significantly different from minimum-length trees, regardless of weighting scheme employed, providing little basis for refuting the monophyly of *Archocentrus*. *A. nigrofasciatum* and *A. septemfasciatum* appear to be closely related sister species, and *A. centrarchus* may be a sister species to these two taxa, although *A. centrarchus* is well differentiated (Fig. 5). Kullander (1996) noted

differences in tooth morphology between *A. centrarchus* and *A. nigrofasciatum*. Two other *Archocentrus* species (*A. sajica* and *A. myrnae*) fall outside this group and do not show any strong tendency to cluster with each other or with other genera. *A. myrnae* has only recently been described as a new species (Bussing, pers. comm.). Our results suggest that these two species may deserve recognition as distinct genera.

Second, the two species of *Caquetaia* (*Ca. krausii* and *Ca. umbifera*) are highly divergent and support for a sister-taxa relationship between these two is tenuous (Figs. 4 and 5). Although the monophyly of *Caquetaia* cannot be refuted using Wilcoxon sign-rank tests, the large genetic distinction between these taxa is not reflected by the current taxonomy, suggesting either that *Ca. umbifera* may be more closely allied with another genus or should be recognized separately in a new genus. Kullander (pers. comm.) noted that *Ca. umbifera* has been included within *Caquetaia* with reservations. Similar results were obtained for the two species of *Parachromis*.

Finally, although the monophyly of species recognized as belonging to the genus *Amphilophus* is not disputed (Figs. 4 and 5), the genetic divergence among some species is similar to the divergence observed between recognized genera (Fig. 5). Furthermore, the genetic distinction among geographic populations of *Am. alfari* can be quite large. For example, between populations from the Pacific slope Barranca drainage and the three, monophyletic Atlantic slope drainages sampled (Sixaola, San Juan, and Matina) there are 57 base differences (approximately 5% sequence difference). This divergence is very similar to the average divergence measured for geminate species of marine fishes separated by the rise of Isthmus of Panama 3–4 mya (Bermingham *et al.*, 1997). Individuals from the Sixaola and San Juan (both Atlantic slope rivers) differ at about 2.5% of the sites in the cytochrome b gene. Given the relatively slow rate of nucleotide substitution in fish mitochondrial DNA relative to "conventional" rate estimates for vertebrates (Martin *et al.*, 1992; Bermingham *et al.*, 1997), these levels of sequence differentiation within species are remarkable and suggest that many of these populations have been isolated for millions of years. Long-term isolation may explain the unique color patterns of *Am. alfari* in the Sixaola drainage (Lopez, 1983). Currently, *Am. alfari* from the Sixaola drainage is being described as a new species, *Am. bussingi* (W. Bussing, pers. comm.), a revision that is supported by genetic and morphological data. We would also argue in favor of recognizing *Am. alfari* from Barranca drainage as a distinct species. Genetic data also support the suggestion that *Am. alfari* (and its sister species *Am. rhytisma*) should not be included in *Amphilophus* (as defined by the type species *froebelii* = *labiatus*) (Bussing and Martin, 1975; Kullander, pers. comm.; Roe *et al.*, 1997).

Overall, we observed considerable genetic divergence among species and the data seem to confirm the growing necessity for taxonomic revision of heroines. Estimates of genetic distinction and relationships afforded by analysis of mitochondrial DNA sequences provide an excellent source of information that, when combined with analysis of morphological characters, should yield a predictive classification. Obviously, a full revision of the taxonomy and systematics of heroine cichlids will have to wait until detailed morphological work and mtDNA sequencing can be accomplished for a broader range of species and genera than were sampled in this study. We are pursuing collaborative studies to this end. Although the systematics of heroine cichlids remain obscure, the data clearly show that heroine cichlids have probably been in Central America since the middle or early Miocene. The star-burst pattern of relationships among most genera suggest that soon after their arrival (from South America or the Caribbean), the ancestors underwent an explosive radiation.

Adaptive Radiation and Convergence

Analysis of mtDNA sequence differentiation among distinctive cichlids suggests an explosive adaptive radiation that appears to have occurred coincident with a middle to late Miocene colonization of Central America. All of the distinctive phenotypes trace their ancestry to the base of the tree, with the exception of the distinctive filamentous algal feeding *Neetroplus*, which originated relatively recently from a scraper-type morphology typical of *Paraneetroplus* and *Hypsophyrs*. The hypothesis of an explosive adaptive radiation is consistent with Myers' view (1966) that prior to the invasion of fishes from South America there was an ostiophysian vacuum in Central America. Presumably, the chance colonization of one (or a few) lineages of cichlids was followed by a sweepstakes-like niche diversification of fishes that filled an ecological vacuum.

Comparison of cichlid ecology and morphology with proposed phylogenetic hypotheses suggests that there has been convergent phenotypic evolution from a generalist-type ancestor (Fig. 7) and suggests multiple origins of adaptations for piscivory and rock-scraping/tearing feeding modes. (This inference is apparent in all of the trees generated from the data.) If defined ecological types are constrained to be monophyletic, the resultant topology is significantly longer than the minimum-length tree (Wilcoxon sign-rank test, $Z = -5.5$, $P = 0.0001$). A few cases deserve attention. First, *Tomocichla tuba* and *Nandopsis* evolved a scraper/tearer morphology independently of the *Paraneetroplus–Neetroplus–Hypsophyrs* clade. Similarity of form suggested a close relationship between *Tomocichla* and the *Paraneetroplus* clade (Bussing, 1985). *Tomocichla* is unique, however, in its almost exclusively frugivorous diet, whereas the other species scrape algae and consume detritus and vegetation (Bussing, 1987;

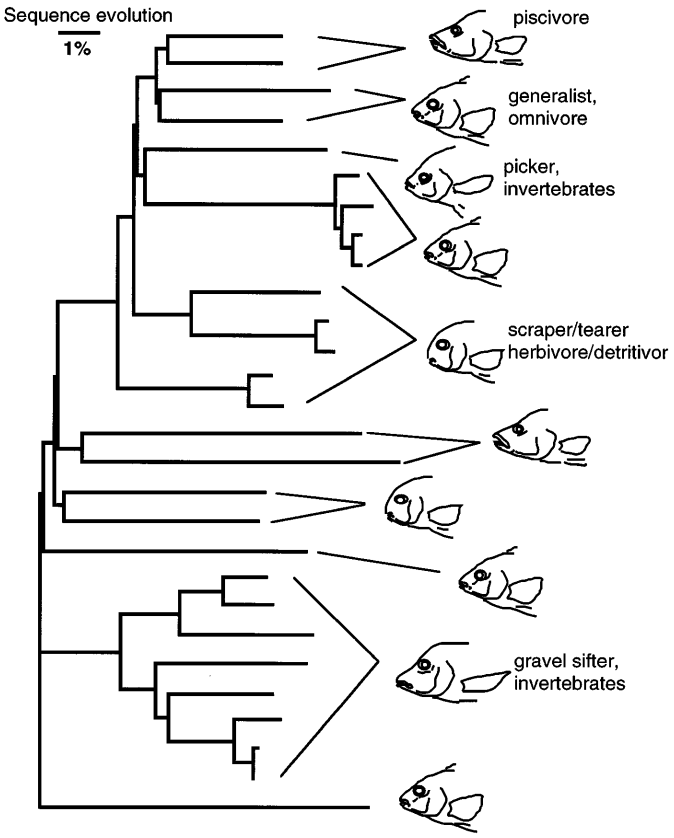

FIG. 7. Neighbor-joining topology (Fig. 5A) with taxon names removed and replaced with drawings of a generalized head form characteristic of the ecomorphology.

Winemiller *et al.*, 1995). Second, piscivory is accompanied by evolution of elongated body form and specialized dentition. Both *Parachromis* and *Caquetaia* possess upper and lower jaw fangs and recurved caniniform teeth (and other morphological features), suggesting a close phylogenetic relationship between these genera (Kullander, pers. comm.). The molecular data clearly show that these genera are distantly related, however, so that character covariation most likely reflects similar selection regimes associated with piscivory.

The hypothesis of multiple episodes of convergent evolution is tentative, pending analysis of additional taxa; however, the hypothesis accords well with studies of African cichlids (Kocher *et al.*, 1993). Additional studies of the behavior and morphology of Central American cichlids are needed to evaluate the extent of convergent evolution and to more fully infer the tempo and the mode of the adaptive radiation.

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